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Rapid identification of *Candida* spp. in peritonitis patients by Raman spectroscopy

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ABSTRACT

This prospective study evaluated Raman spectroscopy for the identification of clinically relevant *Candida* spp. in peritonitis patients. A Raman database was developed by measuring spectra from 93 reference strains belonging to ten different *Candida* spp. Clinical samples were obtained from the surgical department and intensive care unit of a tertiary university hospital. In total, 88 peritoneal specimens from 45 patients with primary, secondary or tertiary peritonitis were included. Specimens were cultured initially on a selective Sabouraud medium that contained gentamicin to suppress bacterial growth. For conventional identification, a chromogenic medium was used for presumptive identification, followed by use of the Vitek 2 system for definitive identification (requiring a total time of 48–96 h). Raman measurements were taken on overnight cultures from Sabouraud–gentamicin medium. Thirty-one samples were positive for *Candida* by culture. Using multivariate statistical analyses, a prediction accuracy of 90% was obtained for Raman spectroscopy, which appears to offer an accurate and rapid (12–24 h) alternative for the identification of *Candida* spp. in peritonitis patients. The reduced turn-around time is of great clinical importance for the treatment of critically ill patients with invasive candidiasis in intensive care units.

Keywords *Candida* spp., identification, Raman spectroscopy, rapid identification method, peritonitis, spectroscopy

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INTRODUCTION

Candida spp. are often referred to as 'emerging pathogens'. *Candida* was considered originally to be an insignificant, transient contaminant, but is now recognised as a serious nosocomial pathogen in non-neutropenic critically ill patients in intensive care units (ICUs) [1–3]. The incidence of *Candida* infections has risen significantly during recent decades, with the greatest increase occurring in surgical services, and especially in patients recovering from abdominal surgery [1,4–7]. The morbidity and mortality associated with these invasive *Candida* infections is striking, with the median ICU stay increased by up to 30 days [8,9] and mortality rates of 30–80% [10–13].

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Several previous studies have assessed the positive effect of early, systemic antifungal therapy on the outcome of ICU patients with invasive *Candida* infections, in terms of a decrease in both morbidity and (attributable) mortality [3,4,11,14–17]. As amphotericin B may have toxic side-effects, fluconazole is often the first choice for prophylactic or empirical antifungal therapy [3,18,19]. However, two of the heralded problems involved in using fluconazole for prophylaxis are the existence of fluconazole-resistant non-*albicans* *Candida* spp. [20], and the possible emergence of fluconazole resistance *de novo* through selective pressure following prolonged use of azoles. Such prolonged use may select for either less susceptible non-*albicans* *Candida* spp., or a shift to fluconazole-resistant *Candida albicans* [21]. Accordingly, rapid identification of significant isolates to the species, or even strain, level is imperative for prompt initiation of appropriate antifungal therapy, since susceptibility data for

the isolate may not be available immediately. Also, the adequacy of the initial, empirical treatment has been proved to influence morbidity and mortality. Changes in therapy following a delay for culture results failed to improve outcome if the initial regimen was inappropriate [22].

Current identification techniques are, at best, blunt instruments with limited sensitivity. A convincing tool for the rapid diagnosis of invasive candidiasis has yet to emerge [23]. However, one possibility lies in the use of vibrational spectroscopic techniques (Raman and infrared spectroscopy), which yield spectra that are molecule-specific. When applied to complex biological samples, such as cells or tissues, the spectra are a summation of the signal contributions of all molecular species, and therefore reflect the overall molecular composition of a sample. Such spectra have been shown to be highly suitable for rapid identification of bacteria [24,25] and yeasts [26,27], because they are reproducible and distinct for different bacterial and fungal species. Previous studies have indicated that the technique might provide sufficient resolving power to enable the discrimination of microorganisms to the strain level, and also to provide information regarding susceptibility to antimicrobial or antifungal agents [26,28]. Vibrational spectroscopy appears to offer many advantages over current routine methods for the identification of *Candida* spp., in that it requires minimal biomass and minimal sample handling, but enables rapid, direct and accurate analysis of samples, with the possibility of automation. Rapid identification of clinically relevant microorganisms from solid culture medium, based on confocal Raman microspectroscopy, has been reported recently. Reproducible Raman spectra were obtained from microcolonies 10–100 μm in diameter after an incubation time of only 6 h [29,30]. In view of these results, the present study aimed to evaluate the feasibility, accuracy and turn-around time of this technique in a prospective clinical study for the identification of *Candida* spp. isolated from patients with peritonitis.

MATERIALS AND METHODS

Database strains

A collection of 93 reference *Candida* strains, comprising ten different *Candida* spp., was used. Strains were either obtained from culture collections or were clinical isolates identified to the species level by conventional identification methods. Strains were stored at −80°C in brain–heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) containing glycerol 10% v/v until use. Before measurements were made, the strains were cultured on Sabouraud–gentamicin medium (Merck, Darmstadt, Germany) for 12–24 h at 30°C. Two independent cultures of each reference strain were used to generate Raman spectra.

Technical procedures of confocal Raman microspectroscopy

Raman spectra were generated as described previously [29,30]. Briefly, with the CaF_2 substrate placed under a microscope (fitted with an 80× near-infrared objective lens; MIR Plan 80x/0.75; Olympus, Amsterdam, The Netherlands), Raman spectra were obtained using a System 1000 Raman microspectrometer (Renishaw, Wotton-under-Edge, UK). Ten spectra, obtained at randomly chosen positions within each smear, were measured using *c*. 100-mW laser light (830 nm) and a signal collection time of 30 s/spectrum.

Statistical analysis

All spectral analyses were performed as described previously [30]. Briefly, the first derivatives of the spectral range from 400 to 1800 cm^{-1} were used to minimise the influence of background signal caused by slight sample fluorescence. The ten spectra collected from each smear were averaged, after which the amount of data was reduced using principal component analysis (PCA), performed with the PLS toolbox (Eigenvector Research Inc., Manson, WA, USA) for Matlab software (Mathworks Inc., Natick, MA, USA). These PCA scores were used for hierarchical cluster analysis (SPSS software; SPSS Inc., Chicago, IL, USA) to generate a dendrogram. Based on the major clusters found in the dendrogram, six linear discriminant analysis models (LDA) were calculated to construct an identification scheme. For LDA, only PCA scores accounting for >1% of the variance in the data set were retained. A two-sided *t*-test was used to individually select those PCA scores that showed the highest significance in discriminating the different microbial groups presented. The number of PCA scores used as input for an LDA model was kept at least two-fold less than the number of spectra in the smallest model group to prevent overfitting in the LDA model. The prediction accuracy of this model was tested using a 'leave-one-strain-out' method; that is, the spectra of all but one strain were used to generate the LDA model [30]. By repeating this procedure, and leaving the spectrum of each strain out in turn, information was obtained on the accuracy and reproducibility of the identification scheme, i.e., whether there was enough discriminating information in the Raman spectra to identify spectra of unknown samples correctly.

Patient sample collection

During 11 weeks in 2001, all patients from the surgical ICU or from the general surgical ward with primary, secondary or tertiary peritonitis were included prospectively. In total, 88 peritoneal specimens were obtained: 55 from 20 ICU patients; 22 from 17 patients hospitalised on general surgical wards; and 11 from eight patients with an infection following continuous ambulatory peritoneal dialysis (CAPD). Specimens from peritoneal fluid or from an intra-abdominal abscess were obtained during a laparotomy. Specimens from CAPD fluid were

obtained directly from the CAPD catheter. Upon arrival in the microbiology laboratory, each specimen was divided into two, with one part used for conventional microbiological identification (the reference method), and the other for identification by Raman microspectroscopy.

Conventional microbiological identification of isolates from patients

For conventional isolation and identification of yeasts, samples were cultured on CHROMagar *Candida* medium (Becton Dickinson). After incubation at 30°C for 48 h, a presumptive identification was made, based on distinctive coloured colonies. A definitive identification was then obtained using the Vitek 2 system (bioMérieux, Lyon, France), with a total turnaround time of 48–96 h.

Raman measurements and identification of isolates from patients

Patient samples were cultured under identical conditions to the database strains (see above). If positive for yeasts, several well-isolated colonies from the overnight cultures were smeared onto a CaF_2 substrate. The smears were dried in a desiccator over drying beads for at least 25 min before Raman measurements were taken.

RESULTS

Representative Raman spectra acquired from database *Candida* strains are shown in Fig. 1. Close inspection of the spectra revealed that there were spectral differences characteristic of the various species. These spectra were subjected to multivariate analysis to calculate six linear discriminant models. Fig. 2 shows the schematic representation of this sequential identification model. The strength of this model based on the training set was evaluated using the 'leave-one-strain-out' method. The prediction accuracy of this model was 87%.

Spectra obtained with patient specimens were analysed with this species identification scheme. In total, 31 (35%) of 88 specimens were positive for *Candida* spp., comprising 30 (55%) of the specimens from ICU patients and one (5%) of the specimens from general surgical ward patients. None of the 11 specimens obtained from a CAPD catheter was positive for *Candida* spp. Of the 31 positive specimens, isolates from 29 specimens were available for further study. Isolates from two specimens, one identified by Raman spectroscopy and one by conventional culture, failed to grow on further subculture.

Conventional and Raman identification of the 29 *Candida*-positive specimens is summarised in Table 1. Single *Candida* spp. were isolated by conventional culture from 20 specimens, while nine specimens contained mixed *Candida* spp. The Raman species identification was obtained by presenting the spectra to models 1–6 of the database sequential identification scheme (Fig. 2). There was a difference between the microbiological and Raman identification outcome in three cases. One *C. albicans* isolate was identified by the Raman technique as *Candida tropicalis*, and another as *Candida dubliniensis* (Fig. 2: model 2). One mixed culture containing *C. albicans* and *Candida glabrata* was misidentified

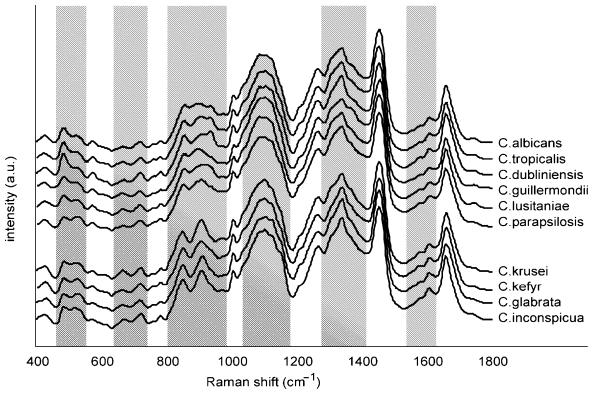

Fig. 1. Representative Raman spectra of ten *Candida* spp. used to establish the *Candida* database in this study. The shaded areas highlight some characteristic differences between the species (a.u. = arbitrary units).

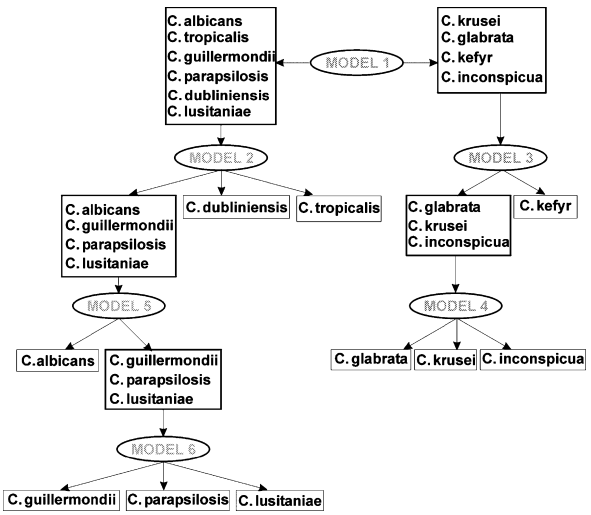

Fig. 2. Schematic representation of the sequential species identification procedure, based on the *Candida* database LDA model 1–6. Spectra of trial specimens to be identified are predicted by using model 1, followed by the next projections.

Table 1. Comparison of the results obtained by conventional microbiological identification and Raman spectroscopy of the *Candida* strains from 29 trial specimens

Conventional identification	Raman identification					
	C. albicans	*C. glabrata*	*C. inconspicua*	*C. tropicalis*	*C. dubliniensis*	Total
C. albicans	13			1	1	15
C. albicans/glabrata	6	2		1		9
C. glabrata		2				2
C. inconspicua			2			2
C. tropicalis				1		1
Total	19	4	2	3	1	29

as *C. tropicalis*. Of the other eight mixed cultures containing *C. albicans* and *C. glabrata*, only *C. albicans* ($n = 6$) or *C. glabrata* ($n = 2$) were identified by Raman spectroscopy. Taking conventional microbiological identification as the reference method, the accuracy of the Raman identification scheme was 90%.

DISCUSSION

The incidence of nosocomial invasive candidiasis has risen during the past decades, with a shift towards the selection of less susceptible *Candida* spp. Indeed, in the USA SCOPE survey of nosocomial *Candida* bloodstream infections, 50% of *Candida* isolates were non-*albicans* spp. [31]. Awareness, especially in intensive care practice, of the growing impact of invasive candidiasis is the first step towards rapid identification and diagnosis. However, the increasing incidence of less susceptible non-*albicans* spp. has created a need for rapid and accurate identification of clinically significant yeast isolates to enable prompt initiation of appropriate antifungal therapy. The adequacy of the initial, empirical treatment has proven to be of paramount significance in terms of morbidity and mortality in critically ill patients with an invasive *Candida* infection [32].

Clinical microbiologists face an important challenge in selecting a system for yeast identification that is both rapid and accurate (high specificity and sensitivity). No method available currently meets these criteria fully. Conventional microbiological identification is based on an extensive series of biochemical assays, following an obligatory culture period sufficient to obtain a biomass of 10^6–10^8 cells [33]. This standard identification method is time-consuming, with an unavoidable turn-around time of 48–96 h. Numerous methods have been developed with the aim of facilitating rapid same-day yeast identification, but most of these systems are designed only to discriminate between two common species or to confirm a presumptive identification [28,34]. A second identification system, such as commercially available yeast identification panels, is often needed for a definitive identification [35,36]. Consequently, up to 4 days may be required before a definitive report reaches the clinician. In addition, the reported accuracies of commercial yeast identification systems vary from 60% to 99% [28,37]. Therefore, a need still remains for rapid and accurate multi-species identification of significant yeast isolates.

The present study investigated the suitability of confocal Raman microspectroscopy for the rapid identification of *Candida* spp., since Raman spectra can be obtained directly from microcolonies on a solid culture medium after culture for only 6 h. Following the development of a new, rapid method for the identification of clinically relevant microorganisms [29], the same method was evaluated for the identification of *Candida* spp. with 42 reference strains [30]. A high degree of accuracy of 97–100% was obtained.

In the present clinical study, the prediction accuracy of the Raman identification method was 90%. One *C. albicans* isolate was identified by the Raman technique as *C. dubliniensis* [38], which shares many phenotypic properties with *C. albicans*, and is often misidentified as *C. albicans* in the microbiology laboratory [28]. However, because of possible resistance of *C. dubliniensis* to azole antifungal agents, it is of great importance to differentiate the two species. Easy-to-perform selective isolation procedures for these closely related species do not exist. Marot-Leblond *et al.* [39] have described an anti-*C. albicans* cell-wall surface-specific monoclonal antibody that might be a candidate for the differentiation of *C. albicans* from *C. dubliniensis*. Tintelnot *et al.* [27] evaluated discriminatory phenotypic markers for *C. dubliniensis*, but concluded that only Fourier transform infrared spectroscopy combined with hierarchical clustering was as reliable as genotyping for discriminating the two species. Future Raman

studies with *C. albicans* and *C. dubliniensis* isolates may reveal the accuracy of the method in discriminating these two related species.

For eight mixed cultures containing *C. albicans* and *C. glabrata*, as identified by culture, only one of these species was predicted by Raman spectroscopy after incubation on Sabouraud medium, presumably because a mixed culture was not detected on this medium and the spectrum of only one *Candida* sp. was measured. This was not counted as a misidentification, although this influences the prediction accuracy, as it was believed that this was not caused by the intrinsic identification capabilities of Raman spectroscopy. Chromogenic media are often used for the recognition and presumptive identification of mixed yeast cultures, and Raman spectra have been measured from CHROMagar medium isolates after only one overnight passage (personal unpublished data). Further studies are required to measure Raman spectra directly from isolates cultured on CHROMagar medium, followed by extended incubation to facilitate the recognition of mixed cultures. Mixed *Candida* infections are uncommon, but not rare, ranging from 9% to 38% of *Candida* infections [12,15,40]. At present, use of chromogenic media is the only method that demonstrates improved detection of yeasts in mixed cultures over traditional media, although only presumptive identifications are obtained.

In the present study, Raman spectra were measured from smears after overnight culture, which is practical in a clinical setting, compared to the microcolonies obtained after growth for 6 h which were used in the pre-clinical trial [30]. Raman measurements from smears are easier and more rapid to perform, but are not as homogeneous as those obtained with microcolonies. In total, Raman spectra were measured from 29 overnight cultures, resulting in a turn-around time of ≤ 1 day, instead of the period of 3–4 days required for conventional identification. Unfortunately, a dedicated Raman spectrometer or universal spectral databases are not yet available commercially. A Raman spectrometer coupled to a microscope is most practical for studies such as those described in the present report, and this combination is available from several commercial suppliers.

In conclusion, Raman spectroscopy appears to offer an accurate and rapid alternative for the identification of *Candida* spp. in ICU patients with peritonitis. Further investigations should aim to optimise the technique for improved detection of mixed *Candida* cultures and to evaluate the impact of this novel identification method on clinical practice and patient outcome in a prospective clinical trial.

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